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Induced and Natural Regulatory T Cells in the Development of Inflammatory Bowel Disease

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Abstract

The mucosal immune system mediates contact between the host, and the trillions of microbes that symbiotically colonize the gastrointestinal tract. Failure to tolerate the antigens within this “extended self” can result in inflammatory bowel disease (IBD). Within the adaptive immune system, the most significant cells modulating this interaction are Foxp3⁺ regulatory T (T_{reg}) cells. T_{reg} cells can be divided into two primary subsets: “natural” T_{reg} (nT_{reg}) cells, and “adaptive” or “induced” T_{reg} (iT_{reg}). Recent research suggests that these subsets serve to play both independent and synergistic roles in mucosal tolerance. Studies from both mouse models and human patients suggest defects in T_{reg} cells can play distinct causative roles in IBD. Numerous genetic, microbial, nutritional, and environmental factors that associate with IBD may also affect T_{reg} cells. In this review we summarize the development and function of T_{reg} cells, and how their regulatory mechanisms may fail, leading to a loss of mucosal tolerance. We discuss both animal models and studies of IBD patients suggesting T_{reg} cell involvement in IBD, and consider how T_{reg} cells may be used in future therapies.

Keywords

induced regulatory T cell; natural regulatory T cell; colitis; Crohn’s disease; Foxp3

Introduction

The gastrointestinal tract is the largest lymphoid organ in the body. This complex network consists of specialized epithelial cells, lymphatic vessels, dendritic cells, lymphocytes, and secondary lymphoid structures. The gut immune system mediates contact between the host and the microbes that symbiotically colonize this mucosal surface (termed the microbiota), while protecting the host against pathological invasion. Exposure to nutritional, microbial, and host antigens in the intestine creates an “extended self” that varies dynamically in this complex environment. Thus, the division between “self” and “non-self” is often indistinct, and multiple regulatory mechanisms have evolved to restrain misdirected immune responses. Failure of these regulatory pathways and/or imbalances in the composition of the

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microbiota can result in pathologic inflammatory processes such as inflammatory bowel disease (IBD), which primarily consists of ulcerative colitis (UC) and Crohn's disease (CD).

Numerous tissues and specialized cells are involved in maintaining mucosal tolerance in the intestine. Paneth cells at the base of the intestinal crypts of the small intestine produce antimicrobial peptides and lectins, limiting direct contact between the epithelia and microbiota.¹ M cells of the follicle-associated epithelium translocate luminal antigens across the epithelium into the dome region of Peyer's patches (PP) and isolated lymphoid follicles (ILF) for uptake and processing by dendritic cells (DCs).² Recent data suggests that a subset of goblet cells in the small intestine may also deliver antigens to DCs.³ The DCs within the gut lamina propria and secondary lymphoid tissues encounter and process the antigen. Alternatively, DC within the lamina propria can sample luminal antigens directly by extending dendrites between tight junctions of the epithelia, into the lumen. After encountering antigen, DCs migrate to T cell zones in the PP or mesenteric lymph node (mLN) to present antigen and activate effector T cells. Activated CD4 effector T cells can stimulate B cells, resulting in IgA-producing plasma cells. IgA plasma cells and activated effector T cells then populate the lamina propria, resulting in a low-grade inflammation that appears to be the normal state of the intestine.²

IBD appears to be a primarily T cell-mediated disease. Indeed, CD4 T cells are responsible for orchestrating the excessive inflammation involved in IBD.^{4, 5} However, T cells also appear to play the largest role in establishing and maintaining immunological tolerance by suppressing excessive or abnormal immune responses. Numerous subtypes of T cells appear to display immunosuppressive function in the gastrointestinal tract. Classical TCR $\alpha\beta$ T cells such as CD8⁺ CTLs and CD4⁺ Tr1 cells exhibit immune suppression in the gut.^{6, 7} In addition, more atypical CD8 $\alpha\alpha$ and TCR $\gamma\delta$ ⁺ T cells have been shown to exhibit protection against mouse models of colitis.^{6, 7} Other innate-like T lymphocytes with invariant TCRs such as iNKT and mucosal-associated invariant T (MAIT) cells may also play immunoregulatory roles in the GI tract. Nonetheless, the most significant of the immunosuppressive T lymphocytes are TCR $\alpha\beta$ CD4⁺ regulatory T (T_{reg}) cells that are characterized by the expression of the transcription factor Foxp3.⁸ This review will focus on these CD4⁺ Foxp3⁺ T_{reg} cells.

Global failure of T_{reg} cell development via Foxp3 deficiency results in the lethal multi-system autoimmune disease immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) that underscores the essential nature of T_{reg} cells in immune regulation. The most commonly affected organ in IPEX is the intestine, highlighting the essential role for T_{reg} cells in the gastrointestinal tract. In addition to IPEX, other genetic deficiencies suggest the importance of T_{reg} in IBD. Mutations in WASP, CD25, and IL-10 all lead to abnormal T_{reg} cell numbers and/or function, and also increase an individual's risk for IBD.⁹ Although these genetic defects suggest a role for T_{reg} cells in human IBD, much of the mechanistic evidence has come from mouse models of disease. Mouse models of IBD can be separated into 4 relatively distinct categories: 1) chemically induced barrier disruption models, 2) spontaneous models due to genetic deficiency, 3) overexpression of inflammatory mediators, and 4) lymphopenic T cell transfer models (Table 1).

Commonly used chemically induced barrier disruption models include dextran sulfate sodium (DSS) and hapten-induced colitis via 2,4,6-trinitrobenzene sulfonic acid (TNBS) and oxazolone. These chemicals disrupt the epithelium, allowing microbial and immunogenic molecules to penetrate and initiate an inflammatory response.^{10, 11} Spontaneous genetic models include IL-10 deficient mice,¹² defective *PPARG* expression in SAMPI/YitFc mice,¹³ and *Mdr1* deficiency.¹⁴ Numerous other genetic deficiencies also lead to intestinal inflammation, but *IL10*, *PPARG*, and *MDR1* are particularly notable in that they play roles

in T_{reg} cell number and/or function and are also human IBD susceptibility loci.^{11, 15–17} Other mouse models utilize gene overexpression to model intestinal inflammation. Such models include TNF- α overexpression in TNF ^{Δ ARE} mice and STAT4 overexpression.¹¹

Perhaps the most compelling data on the role of T_{reg} cells in IBD has come from the T cell transfer model of colitis. In this model, naïve CD4⁺ T cells, depleted of T_{reg} cells, (typically CD45RB^{hi}) are adoptively transferred into mice lacking B and T lymphocytes (SCID or RAG-deficiency). These effector T cells proliferate and become activated in response to bacterial antigens in the intestine, resulting in inflammation and colitis. Disease can be both prevented and treated via elimination of the microbiota or co-transfer of T_{reg} cells.^{18, 19} This model offers the distinct advantage that the pathogenic and regulatory T cell subsets can be genetically targeted independently. Thus, several important inflammatory and suppressive mechanisms have been identified through transfer of T_{reg} or effector T cells (T_{eff}) from mutant mice.⁶

Numerous studies utilizing mouse models support a role for T_{reg} cells in IBD. In barrier models T_{reg} cells localize to the intestine and mLN in acute disease, and an absence of T_{reg} cells exacerbates disease.^{6, 20} A mild breach of the intestinal barrier via ethanol in the absence of TNBS does not result in colitis, suggesting regulatory responses predominate.⁶ Many genetic models of spontaneous IBD involve genes affecting T_{reg} function, and in treatment models T_{reg} cells home to the intestine to resolve inflammation.^{6, 21} Two subsets of T_{reg} cells have been described, “natural” T_{reg} (nT_{reg}) cells and “induced” or “adaptive” T_{reg} (iT_{reg}) cells. Both nT_{reg} and iT_{reg} subsets are characterized by the expression of Foxp3, and Foxp3 expression is necessary for their overall fate and function.^{8, 22} The nT_{reg} and iT_{reg} subsets are largely distinguished by their developmental origin and appear to play non-redundant roles enforcing gastrointestinal tolerance.

Natural regulatory T cells

“Natural” T_{reg} (nT_{reg}) cells arise as a discrete and largely stable lineage originating in the thymus. Foxp3⁺ cells are first detectable in a small fraction of CD4⁺ CD8⁺ double positive thymocytes, and are subsequently more frequent in CD4⁺ single positive thymocytes.⁸ The nT_{reg} subset exhibits a TCR repertoire that is distinct from Foxp3[−] conventional T cells (T_{conv}) and from iT_{reg} cells. Data shows that the TCRs of nT_{reg} cells may have increased affinities for self-peptides.^{23–26} Moreover, mutations in MHC and TCR signaling suggest that a strong TCR signal is required for nT_{reg} development. TCR signaling activates the NF- κ B pathway, and several conditional mutations in NF- κ B members show nT_{reg} defects.²⁷ NF- κ B family member c-Rel binds directly to the conserved non-coding sequence 3 (CNS3) region of the Foxp3 promoter, providing a link between TCR signaling and Foxp3 expression.²⁸

TCR-ligand affinity alone does not determine Foxp3 expression.²⁸ nT_{reg} cells also require IL-2 for their development and maintenance. In fact, regulatory T cells were first identified by their elevated expression of the high-affinity IL-2 receptor CD25 (IL-2R α). Mice lacking IL-2 signaling via antibody neutralization or genetic deficiency of IL-2 or IL-2 receptors show nT_{reg} deficiencies and spontaneous autoimmune disease including IBD.^{29–32} CD25 deficiency in human patients also results in an IPEX-like disease, supporting the importance of IL-2 signaling in nT_{reg} development.³³ Downstream signaling from IL-2 receptors is mediated through STAT5, and STAT5 binds to the conserved non-coding sequence 2 (CNS2) enhancer region of the Foxp3 gene. This suggests that IL-2 signaling directly promotes the initial expression and/or maintenance of Foxp3 to support nT_{reg} development.²⁷

In addition to IL-2 signaling, normal T_{reg} development in the thymus requires autophagy. Transplant of autophagy-deficient thymi from Atg^{-/-} mice results in abnormal T cell selection and spontaneous colitis.³⁴ This suggests that a subset of intestinal auto-antigens may require autophagy for proper presentation and deletion in the thymus. In fact, autophagy genes are a commonly identified class of IBD susceptibility alleles. Thus, there may be a role for autophagy defects in clinical disease, though T_{reg} specificity and function in IBD patients with autophagy mutations has not been investigated.

Adaptive or induced regulatory T cells

Whereas nT_{reg} cells are defined by Foxp3 expression that originates during thymic T cell development, “adaptive” or “induced” T_{reg} (iT_{reg}) cells are generated during the course of an immune response from naive T_{conv} cells in the periphery. In healthy mice, the majority of peripheral Foxp3⁺ cells in the spleen and lymph nodes are thymically-established nT_{reg} cells. In contrast, the iT_{reg} subset of T_{reg} cells make up a large fraction of T cells in the lamina propria (LP) and gut-associated lymphoid tissue (GALT) of the intestine.^{34, 35} The formation of iT_{reg} cells involves the peripheral activation of T_{conv} cells in the presence of TGF-β1 (Figure 1). This conversion can also be performed in culture, resulting in Foxp3⁺ *in vitro* iT_{reg} cells.³⁶ These *in vitro* iT_{reg} cells may serve as cell-based therapies for autoimmune diseases such as IBD since they can be derived in large numbers from the patient’s own cells, and can potentially be enriched for relevant antigen specificities. Indeed, clinical trials utilizing *in vitro*-derived iT_{reg} cells as a new therapy are currently under way.³⁷

In vivo, antigen exposure and/or lymphopenia can result in Foxp3 expression in up to 15% of antigen experienced T_{conv} cells.³⁸ These *in vivo* iT_{reg} cells appear to be particularly prevalent at mucosal surfaces where tolerance must be expanded to antigens within the colonizing microbiota.^{27, 39} In addition to the microbiota, ingested food contains a plethora of antigens. Immune responses to these dietary molecules can be actively suppressed by inducing oral tolerance. Antigen feeding experiments identified a TGF-β1-producing CD4⁺ T cell population in the LP and GALT coined “Th3” that was important in oral tolerance.⁴⁰ These studies were performed prior to the discovery of Foxp3, and subsequent studies have shown that most “Th3” cells express Foxp3 and are *de facto* iT_{reg} cells. However, a small fraction of CD4⁺ TGF-β1⁺ Foxp3⁻ Th3 cells may yet exist.⁴¹

Similarly to *in vitro* iT_{reg}, the peripheral induction of Foxp3 *in vivo* appears to be dependent upon TCR activation in the presence of TGF-β1.^{36, 42, 43} TGF-βR signaling leads to phosphorylation of SMAD molecules and the binding of SMADs to the CNS1 enhancer element in the *Foxp3* locus.⁴⁴ This association together with the binding of other transcription factors leads to the TGF-β1-mediated activation of *Foxp3* transcription, which drives differentiation of the iT_{reg} lineage.^{28, 45} The vitamin A metabolite retinoic acid (RA) further increases *Foxp3* induction.⁴⁶

In vivo induction of Foxp3 seems to be particularly important in the gut. In fact, the GI tract is a TGF-β1 rich environment, LP and GALT dendritic cells (DCs) produce RA, and RA drives gut tropism in T_{reg} cells.^{6, 47} Additionally, APCs derived from the intestine, including CD103⁺ DCs and CD11b^{hi} CD11c⁻ macrophages preferentially promote iT_{reg} development in comparison to spleen or peripheral lymph node derived APCs.^{6, 27, 39} These data strongly support a role for induction of iT_{reg} and iT_{reg}-mediated suppression in the gastrointestinal environment.

Distinguishing nT_{reg} and iT_{reg} cells

There are few mechanisms for differentiating nT_{reg} and iT_{reg} cells. Most molecules showing increased expression on T_{reg} cells (including CD25, CTLA4, and GITR) appear to be targets of *Foxp3* transcriptional activation, and are thus expressed similarly between iT_{reg} and nT_{reg} cells. Also, both nT_{reg} and iT_{reg} cells suppress immune responses using similar antigen-dependent and antigen-independent mechanisms.² Other cell types can also express ostensible T_{reg} markers, further confusing the discrimination between T_{reg} subsets.^{48, 49}

Helios is one molecule that has been suggested to differentiate nT_{reg} and iT_{reg} cells. Helios is a transcription factor that is expressed at elevated levels in nT_{reg} but not iT_{reg} cells.⁵⁰ However, genetic deficiency of Helios does not appear to affect T_{reg} differentiation and function.⁵¹ Subsequent studies have suggested that Helios is a marker of activation and proliferation that can be expressed on both T_{reg} and T_{conv} cells.⁵² Furthermore, in some studies only a small fraction of *in vivo*-derived iT_{reg} cells lacked Helios expression²⁴ and both *in vitro* and *in vivo*-derived iT_{reg} could express Helios in response to APC stimuli.⁵³

Neuropilin-1 (Nrp-1) is another marker that may differentiate nT_{reg} and iT_{reg} cells. Recent studies suggest that Nrp-1 is limited to the nT_{reg} subset.^{54, 55} The iT_{reg} cell subset expresses only low levels of Nrp-1 after *in vitro* conversion via TGF-β1. *In vivo* iT_{reg} cells differentiated via oral or intravenous antigen delivery, exposure to a peripherally-expressed antigen, or lymphopenia-induced proliferation also lack Nrp-1 expression. Interestingly, Nrp-1 expression differences are largely maintained, even when cells are activated.^{54, 55} Conversely, iT_{reg} cells differentiated under inflammatory conditions in the lung or CNS appear to gain Nrp-1 expression.⁵⁴ Thus, it will be interesting to examine Nrp-1 expression in mouse models of IBD. Unfortunately, Nrp-1 does not appear to be expressed by human PBMCs, so the utility of this marker for clinical studies may be limited.⁵⁶

Differences in the methylation status of the *Foxp3* genetic locus are a more universally accepted difference between the nT_{reg} and iT_{reg} subsets. Thymus-derived nT_{reg} cells have demethylated CpG motifs in the T_{reg} cell-specific demethylation region (TSDR) of conserved noncoding sequence 2 (CNS2) in *Foxp3*, whereas the TSDR of iT_{reg} cells is only partially demethylated.⁵⁷ Heritable and stable *Foxp3* expression are linked to TSDR demethylation. The factors that influence the demethylation status of the TSDR in iT_{reg} cells are unknown and the level of demethylation varies depending on the nature of the iT_{reg} differentiation.^{24, 58} Unfortunately, detection of *Foxp3* TSDR methylation must be performed on the genomic DNA of large pools of cells, and thus cannot differentiate nT_{reg} and iT_{reg} cells on a per cell basis as with surface markers.

Since markers that differentiate nT_{reg} and iT_{reg} are controversial, current studies often identify all peripheral *Foxp3*⁺ T cells as “nT_{reg}”. This practice complicates the precise determination of nT_{reg} and iT_{reg} cell identity and function, because the peripheral *Foxp3*⁺ T cell pool undoubtedly contains some fraction of T_{reg} cells that induced *Foxp3* after leaving the thymus. Thus, the population of cells identified as “nT_{reg} cells” are likely contaminated with *in vivo*-derived iT_{reg} cells. The fraction of iT_{reg} in a typical peripheral T_{reg} pool has been estimated by tracking *Foxp3*[−] T_{conv} cells that upregulate *Foxp3* expression *in vivo*. In these approaches, congenically marked *Foxp3*[−] T_{conv} cells are transferred into lymphopenic, lymphoreplete, or *Foxp3*-deficient hosts. Using these transfer approaches, the overall fraction of iT_{reg} cells in the peripheral T_{reg} pool appears to be 4–15%, depending on the system used. As noted above, higher frequencies of iT_{reg} cells are generally present in the mLN and GALT than the peripheral lymph nodes and spleen, supporting a role for iT_{reg} cells at mucosal surfaces.^{24, 38, 59}

The size of the iT_{reg} pool was also estimated in Carma-1 deficient mice, which lack thymically-derived nT_{reg} cells but develop iT_{reg} cells in the periphery.³⁴ The overall frequency of Foxp3⁺ cells in the periphery of Carma-1^{-/-} is 3–4% of that in wild-type mice. Since Carma-1^{-/-} mice only have iT_{reg} cells yet have a higher threshold for TCR activation, 3–4% likely represents the low end of the iT_{reg} contribution to the peripheral T_{reg} pool, in agreement with transfer studies. Additionally, compared to the spleen, Foxp3⁺ cells in these mice are twice as frequent in the mLN and ten times as frequent in the LP, further supporting an increased frequency and role for iT_{reg} development at these sites.³⁴ Thus, when considering most studies, it appears that the typical contribution of the iT_{reg} subset to the peripheral T_{reg} pool ranges from 3 to 15% and is highly context-dependent. Consistently, the contribution of iT_{reg} cells increases greatly in the intestine, favoring a significant role for iT_{reg} cells in the control of IBD.

Due to the lack of a specific surface marker, it is currently difficult to identify human *in vivo*-derived iT_{reg} cells, although it is reasonable to assume that the iT_{reg} cell subset exists. Human T cells share a capacity for the *in vitro* differentiation of iT_{reg} cells and have similarly increased numbers and frequency of T_{reg} cells in mucosal locations. One important distinction between mice and humans is that activated non-regulatory human T_{conv} cells transiently express FOXP3 at low levels. Accordingly, human FOXP3⁺ T cell populations are subdivided as a resting/naive T_{reg} cell subset that is CD25^{hi}CD45RA⁺FOXP3^{hi}, an effector T_{reg} cell subset that is CD25^{hi}CD45RA⁻FOXP3^{hi}, and a non-regulatory cytokine-secreting FOXP3⁺ subset that is CD25^{hi}CD45RA⁻FOXP3^{lo}.⁶⁰ Additional markers such as low expression of CD127, and increased expression of CD39, MHCII, GITR, and CTLA-4 further distinguish human T_{reg} cells.⁶¹

Mechanisms of T_{reg} suppression in IBD

T_{reg} cells utilize numerous effector mechanisms to suppress immune responses, targeting both the innate and adaptive arms of the immune system. Interestingly, no one effector mechanism or pathway has been shown to be both necessary and sufficient for all T_{reg}-mediated suppressive activities. There is a great deal of functional redundancy between T_{reg} suppressive pathways, which results in compensatory mechanisms in targeted T_{reg} cells and the need for a very strong inflammatory stimulus to differentiate phenotypes. Also, early deletion of genes involved in T_{reg} function often leads to systemic immune issues before intestinal involvement can be detected.⁶ Studies of IBD are particularly sensitive to slight disparities in the colonizing microbiota between mouse colonies and differing mouse strains, leading to inconsistent results. Despite these difficulties, several effector mechanisms for T_{reg}-mediated suppression of IBD have been described (Table 2). In general they can be divided into two categories 1) immunoregulation through consumption or production of soluble mediators (primarily cytokines) and 2) immunoregulation via direct interaction with APCs or T_{eff} cells.

Among the cytokine-based approaches, TGF-β1 and IL-10 are the best studied. TGF-β1 is a pleiotropic cytokine produced by many immune and non-immune cells. TGF-β1 controls cell proliferation and differentiation in numerous tissues throughout the body. In particular, TGF-β1 plays an important role in the extra-thymic induction of Foxp3 in iT_{reg} cells. In addition, T_{reg} cells themselves produce large amounts of TGF-β1. Global deficiency of TGF-β1 results in a lethal multi-organ lymphoproliferative disease similar to Foxp3 deficiency. Since TGF-β1 deficient mice die at weaning, analysis of gut-specific effects of TGF-β1 difficult.

The intestine appears to be a particularly TGF-β1 -rich environment and apoptotic cells stimulate TGF-β1 release.⁶² The high cell turnover and/or acute epithelial damage in the

intestine may contribute to the secretion of TGF- β 1 to prevent excessive immune responses in the chronically immune-stimulated intestinal environment. However, much of the TGF- β 1 in the intestine is present in an inactive form that must be activated by integrins. Mice deficient in α_v or β_8 TGF- β 1-activating integrins in APC populations develop spontaneous colitis, suggesting DCs and perhaps other APCs must activate TGF- β 1 in the GALT.^{6, 34} This activated TGF- β 1 likely has direct effects on T lymphocytes since depletion of T cells prolongs the survival of TGF- β 1 deficient mice.^{63–65} Further, T cell-specific deletion of TGF- β -receptor II results in a similar autoimmune phenotype to global deficiency of TGF- β 1.^{66, 67} In the T cell transfer model of colitis, a TGF- β 1 blocking antibody eliminates the ability of a T_{reg}-containing CD45RB^{low} pool to prevent colitis.⁶⁸ In a similar transfer model, transferred T cells expressing an endogenous inhibitor of TGF- β 1, Smad7, were unable to be suppressed by co-transferred T_{reg}.⁴² This suggests that T_{reg}-produced TGF- β 1 has direct immunosuppressive effects on colitogenic T_{eff} cells, and/or that TGF- β 1-driven differentiation of iT_{reg} is necessary for prevention of intestinal inflammation.

IL-10 is a potent anti-inflammatory cytokine produced by both non-immune and immune cells including T_{reg} cells. IL-10 has particular significance in IBD because IL-10 and IL-10R2 deficient mice do not develop lethal systemic autoimmunity, as seen in TGF- β 1 deficient mice, but instead develop colitis.^{12, 69} Established intestinal inflammation in mice can be ameliorated via treatment with recombinant IL-10 protein, IL-10 expressing transgenic T cells, or intestinal bacteria engineered to produce IL-10.^{70–72} Significantly, the *IL10* gene is a susceptibility locus for human ulcerative colitis, and patients with mutations in the IL-10 receptor develop IBD at an earlier age and with higher penetrance than IPEX patients.^{73, 74} T_{reg} cell-specific deletion of IL-10 results in a similar, though less severe intestinal inflammation than global IL-10 knockout mice.⁷⁵ In the T cell transfer model of colitis, T_{reg}-produced IL-10 is particularly necessary in the presence of triggering microorganisms such as *Helicobacter hepaticus*.^{76, 77} These results suggest that IL-10 production by both T_{reg} and non-T_{reg} cells is important in maintenance of tolerance in the GI tract, depending on the inflammatory context.

Tr1 cells are one other potential source for IL-10 in the intestine. Tr1 cells produce high levels of IL-10 and require IL-10 for their differentiation. Tr1 cells seem to share other suppressive pathways with T_{reg} cells, but Tr1 cells are characterized by their lack of Foxp3 expression.⁶ In the colon LP, Foxp3⁺ T_{reg} cells are the predominant IL-10 producing T cells. However, in the small intestine, the majority of IL-10-producing intraepithelial lymphocytes (IEL) do not express Foxp3, suggesting a potential role for Tr1 cells.⁷⁸ Since Tr1 cells lack a specific marker, little is known about this population. However, the prevalence of Foxp3[−] IL-10-producing T cells in the intestine, and less severe disease in T_{reg} cell-specific IL-10 knockout mice suggests that Tr1 cells may also contribute to IL-10-mediated suppression.

Another immunosuppressive cytokine secreted by T_{reg} cells is IL-35. IL-35 is a newly discovered heterodimeric cytokine consisting of subunits IL12p35 and Ebi3. IL-35 is preferentially produced by T_{reg} cells and suppresses T cell proliferation *in vitro*. In the T cell transfer model of colitis T_{reg} cells deficient in either IL12p35 or Ebi3 showed decreased ability to ameliorate established disease.⁷⁹ In addition, mice deficient for Ebi3 were more susceptible to EAE, and both Ebi3^{−/−} and IL-12p35^{−/−} mice showed increased airway hyperresponsiveness, suggesting T_{reg}-produced IL-35 may play roles in numerous tissues.^{80, 81}

Pericellular adenosine is another soluble immunoregulatory molecule utilized by T_{reg} cells. Both mouse and human T_{reg} cells express surface bound enzymes such as CD39 and CD73 that convert proinflammatory extracellular ATP into the immunosuppressive nucleoside adenosine. Adenosine produced by T_{reg} cells binds adenosine receptors such as A2A on DCs

and T_{eff} cells to decrease their activation and function.^{82, 83} Pericellular adenosine appears to play a critical role in intestinal immune tolerance. In the T cell transfer model of colitis, wild-type T_{reg} cells could not suppress disease induced by A2A-deficient colitogenic T cells.⁸⁴ Treatment with A2A agonists also ameliorated a spontaneous mouse model of ileitis.⁸⁵

In addition to producing cytokines, T_{reg} cells can also cause immunosuppression through cytokine deprivation. T_{reg} cells express elevated levels of numerous cytokine receptors including the high affinity IL-2 receptor CD25.^{8, 22, 86} This increased expression of cytokine receptors is hypothesized to allow T_{reg} cells to outcompete T_{eff} cells for survival signals, causing T_{eff} cell apoptosis. In the T cell transfer model of IBD, transferred T_{reg} cells increase apoptosis in colitogenic T_{eff} cells. *In vitro* provision of exogenous IL-2 or other common gamma chain cytokines decreases T_{eff} cell apoptosis in T_{reg}/T_{eff} co-cultures.⁸⁷ The Bcl-2 family member Bim signals apoptosis in response to cytokine deprivation. T_{eff} cells deficient in Bim are resistant to T_{reg}-driven apoptosis both *in vitro* and in the T cell transfer model of colitis, suggesting T_{eff} cells are undergoing apoptosis due to an absence of cytokine-derived survival signals.⁸⁷ But, a role for cytokine consumption by T_{reg} cells *in vivo* is difficult to prove. Since T_{reg} cells could utilize other mechanisms to initiate termination of the immune response, survival cytokines would be expected to subsequently decrease as a result of the decreasing immune response.⁶

T_{reg} cells also directly interact with other immune cells to mediate immune suppression. The increased apoptosis of T_{eff} cells via T_{reg} cells may not only be due to cytokine deprivation, but may also be due to direct cytotoxic effects of T_{reg} cells on T_{eff} cells. The direct killing of numerous cell populations including CD4⁺ T_{eff}, CD8⁺ CTL, and APCs by T_{reg} cells via perforins and granzymes is well established *in vitro*.^{88–90} T_{reg}-mediated killing of APCs is a particularly intriguing effector mechanism since this regulatory approach could yield antigen specificity through the TCR/MHCII interactions of these two cell types. The *in vivo* evidence for cytotoxicity of T_{reg} cells is more limited. In a mouse model of transplantation tolerance, granzyme B expression by T_{reg} cells and granzyme targeting in T_{eff} cells was important for maintenance of the graft.⁹¹ In addition, T_{reg} cells were shown to control CD8⁺ T cell responses to lung infection in a granzyme B-dependent manner.⁹² The extent to which cytotoxicity plays a role in T_{reg} cell suppressive function in IBD is currently unknown. Still, it is interesting to note that the increased apoptosis of effector populations through both T_{reg}-mediated cytokine deprivation and cytotoxicity could result in increased TGF-β1 production, further amplifying the regulatory response.

T_{reg} cells also express numerous surface receptors that confer suppressive activity. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is foremost among these receptors. CTLA-4 is a high-affinity inhibitory receptor related to CD28. CTLA-4 has greater affinity for its B7 ligands than CD28. This increased affinity seems to increase the duration of time T_{reg} cells engage with APCs, and modulate the APC function and maturation. In fact, both *in vitro* and intravital imaging suggests that these stable T_{reg}-APC contacts limit access of T_{conv} cells to APCs, thus limiting T_{eff} cell activation.^{93, 94} In the T cell transfer model of colitis, CTLA-4 blockade limits protection by T_{reg} cells. Also, CTLA-4 deficient nT_{reg} cells can prevent colitis induced by wild-type naïve T cells, but not disease induced by CTLA-4-deficient T cells.⁹⁵ This suggests a role for CTLA-4 on nT_{reg} and also T_{conv} and/or *in vivo* iT_{reg} cells. T_{reg} cell-specific CTLA-4 deficiency leads to a lethal autoimmune disorder. However, this lethality occurs much later than in Foxp3 deficiency, suggesting other T_{reg} cell effector mechanisms can temporarily compensate for the absence of CTLA-4.⁹⁶

CTLA-4 also appears to play a particularly important immunoregulatory role in the human intestine. Anti-CTLA-4 (ipilimumab or tremelimumab) treatment increases the immune

response to some cancers by presumably decreasing T_{reg} cell function. However, data from over 4000 patients shows that these treatments can result in potentially lethal colitis.^{34, 97} In addition, treatment with CTLA4-Ig (abatacept) has shown negative effects including new-onset UC in arthritis patients, and exacerbation of symptoms in CD. These results suggest that CTLA-4 may not only block B7 molecules to promote regulation, but that CTLA-4 signaling may be required for T_{reg} cell function in the gut.⁹⁸

T_{reg} cells also express several other receptors that may play a role in immunoregulation. Lymphocyte Activation Gene-3 (LAG-3) is a homologue of CD4 that binds with high affinity to MHCII on the surface of APCs. This binding inhibits DC maturation and leads to more tolerogenic DCs.⁹⁹ T_{reg} cells are one of few cell types that express both programmed death (PD) receptors and ligands. Expression of PD-L1 and PD-1 may promote the differentiation of iT_{reg} cells. PD-L1 and PD-1 expressed by T_{reg} cells may also promote stable contacts between T_{reg} cells and APCs, and modulate APC function, similar to CTLA-4 and LAG-3.¹⁰⁰ These T_{reg} -APC contacts may be maintained and enhanced via T_{reg} cell expression of Nrp-1. T_{reg} cells preferentially express additional receptors such as galectins, GITR, and OX40, but their contribution to T_{reg} effector function is relatively unknown.¹⁰¹

Interestingly, many of these receptors have the potential to interact with both APC and T_{conv} cells to regulate immune responses. Several studies suggest T_{reg} cell suppression may be more important in the lymph nodes than the site of inflammation. In the T cell transfer model of colitis, deletions of gut-homing integrins such as CD103 (αE) shows that migration to the intestine is necessary only in the APC population for T_{reg} to mediate suppression. In addition, T_{reg} cells must express lymph node homing receptors to suppress colitis. This data argues that in the T cell transfer model of colitis T_{reg} cells mediate most of their suppressor function through interactions with gut-homing DCs in the lymph node.^{102–105}

Independent and synergistic roles of nT_{reg} and iT_{reg} cells in mucosal tolerance

It is becoming clear in mouse models of IBD that both nT_{reg} and iT_{reg} cells are necessary for protection and perhaps also treatment of disease.³⁸ However, how the nT_{reg} vs iT_{reg} cell responses are calibrated is currently unknown. TCR repertoire and T_{reg} differentiation studies suggest that the dual requirement for nT_{reg} and iT_{reg} cells may be due to differing TCR specificities between the two subsets of T_{reg} cells. In particular, it seems that nT_{reg} cells are necessary to confer tolerance to self antigens, whereas iT_{reg} cells are induced at mucosal surfaces to confer tolerance to antigens in food and to the microbiota that makeup the “extended self”. Interestingly, IBD patients have increased immunoreactivity to both self and microbial antigens suggesting important roles for both nT_{reg} and iT_{reg} subsets and their corresponding specificities in human patients as well.^{106, 107}

The iT_{reg} subset develops after peripheral activation of T_{conv} cells in the presence of TGF- β 1. However, when levels of the pro-inflammatory cytokine IL-6 are also high, TCR and TGF- β 1 signaling results in upregulation of ROR γ t and development of pro-inflammatory Th17 cells that express the cytokine IL-17 (Figure 1). Thus, iT_{reg} and Th17 cells both arise from T_{conv} cells in the presence of TGF- β 1. In fact, iT_{reg} and Th17 cells may share a common Foxp3⁺/ROR γ t⁺ precursor.¹⁰⁸ Under normal conditions, Th17 cells recruit neutrophils to combat infection by fungi and extracellular bacteria. However, these cells are also associated with numerous autoimmune diseases, including IBD. In fact, increased levels of characteristic Th17 cytokines are found in biopsies from IBD patients.⁴⁸ The role of Th17 cells in IBD is unclear though, as several studies have shown them to be both pathogenic

and protective.³⁹ Mouse studies have disagreed, in particular, on the role of IL-17. Disease can be exacerbated, ameliorated, or unaffected by the absence of IL-17 depending on the method of depletion and whether the IL-17A or IL-17F isoform was targeted.⁴⁸

In addition to IL-17, Th17 cells produce IL-21 and IL-22. IL-21 appears to be largely pro-inflammatory, and promotes further amplification of additional Th17 cells. IL-21 expression is upregulated in lesions of IBD patients.¹⁰⁹ In contrast to the other Th17 cytokines, IL-22 appears to be protective in IBD. IL-22 promotes intestinal barrier integrity and prevent microbes from breaching the epithelium. IL-22 increases cell proliferation and survival of the epithelium, upregulates production of antimicrobial peptides (AMP) such as defensins, and stimulates mucus production.⁴⁸

The Th17 cell phenotype is maintained through binding of IL-23 to Th17-expressed IL-23R. IL-23 appears to be necessary for the development of IBD in several mouse models, suggesting a pathogenic role for Th17 cells.^{77, 110–112} In contrast, one study suggests that IL-23 deficient mice have increased susceptibility to colitis induced by TNBS barrier disruption, which agrees with the role of Th17-produced IL-22 in barrier maintenance.¹¹³ Genome-wide association studies identified IL-23R as one of the genes most strongly associated with both UC and CD in human patients.⁵ One allele of IL-23R (R381Q) is protective for IBD. This mutation results in decreased IL-23R signaling and thus decreased numbers of Th17 cells.¹¹⁴ Since this allele confers about three-fold protection from CD, it appears that Th17 cells may play largely pathogenic roles in human IBD.⁵

Therefore, although iT_{reg} and Th17 cells may share receptor specificities, developmental precursors, and differentiation signals, they have differing impacts on immune responses. In fact, the increased methylation of the *Foxp3* locus of iT_{reg} cells versus that of nT_{reg} cells suggests that the iT_{reg} subset may serve as a more flexible lineage, able to adapt to the antigenic or inflammatory environment. Determining whether iT_{reg} cells can downregulate *Foxp3* expression, becoming so-called “ex-iT_{reg}”, and revert to alternate fates such as Th17 is an active area of research. It is likely that control of the iT_{reg}/Th17 balance and the stability of iT_{reg} are key immune regulatory checkpoints.^{115, 116} Thus, iT_{reg}/Th17 conversion may control regulatory versus inflammatory responses to extended self and foreign antigens, while nT_{reg} cells control responses to self-antigens in the intestine (Figure 1).

A dual requirement for both nT_{reg} and iT_{reg} cells in mucosal tolerance has been supported through several studies. In the T cell transfer model of colitis, disease is much more severe when induced with T_{conv} cells that have an inability to become *in vivo*-derived iT_{reg} cells.³⁸ Mice with established colitis can be successfully treated with the transfer of nT_{reg} cells, but only if the colitogenic T_{conv} population can induce *Foxp3*. In mice lacking *in vivo* iT_{reg} cells, co-transfer of nT_{reg} cells and *in vitro* iT_{reg} can cure disease, suggesting a requisite role for these cells in reversing IBD.³⁸ Further studies demonstrated differences in TCR repertoire between the subsets and suggested the primary role for iT_{reg} cells is to expand the antigen diversity of immunoregulatory responses.²⁴ Though nT_{reg} and iT_{reg} cells appear to utilize similar effector mechanisms, iT_{reg} cells may serve as a flexible lineage able to expand immune regulation to antigens not expressed during thymic nT_{reg} development.¹¹⁷ The ability of iT_{reg} cells to aid in peripheral tolerance appears to be particularly important at mucosal interfaces, where the majority of iT_{reg} cell specificities are to antigens derived from the microbiota.¹¹⁸

Effects of the microbiota on T_{reg} cells

The microbiota have co-evolved with the host to aid in host metabolism, and also play significant roles in host protection and immunity. The microbiota provide signals leading to

the maintenance of the epithelium, and stimulate production of antimicrobial peptides and lectins, thus preventing breach of the initial immune barrier.^{27, 119} The microbiota also directly interact with the innate immune system and aid in adaptive immunity by promoting the development of Peyer's patches and isolated lymphoid follicles in the gut, as evidenced by GF mice lacking these structures.¹²⁰ In turn, the host immune system limits microbial access to the epithelium via the production of antimicrobial products and the secretion of mucus and IgA.¹²¹

IBD patients exhibit abnormal shifts in the taxa of their microbiota. It is not known whether this abnormal bacterial growth is a direct cause of IBD, or a side effect of increased inflammation in the intestine. Nonetheless, probiotics, antibiotics, and fecal diversion have all shown some beneficial effects in IBD.^{39, 122, 123} Thus, it seems increasingly likely that in IBD there are abnormalities in both the microbiota and the immune response, and that these dual abnormalities may be related.

The T_{reg} cell compartment seems to be particularly sensitive to changes in the microbiota. TGF- β 1 and IL-10 expression by T_{reg} cells is necessary for their suppressive capabilities, but only in the presence of an activating microbiota.³⁴ T_{reg} cells from GF mice are generally less suppressive, and express less Foxp3 than T_{reg} cells from mice under normal conditions.^{124–126} Though, one study has demonstrated significant IBD suppression mediated by a T_{reg}-containing population from GF mice.¹²⁷ The presence of colonizing microbes may also affect the prevalence of T_{reg} cells in the GALT. T_{reg} cells numbers are decreased in the colons of GF mice, but are increased in the small intestine.²⁷ Further, T_{reg} cell numbers in the small intestine LP increase in response to decreasing bacterial load via vancomycin treatment.¹²⁸ Consistent with these findings, TLR9-deficient mice have increased numbers of T_{reg} cells in the small intestine, suggesting that inflammatory signals from bacterial DNA plays a role in inhibiting iT_{reg} cell differentiation or T_{reg} cell proliferation.¹²⁹

Mouse studies of IBD have identified *Helicobacter hepaticus* and segmented filamentous bacteria (SFB) as two particularly pro-inflammatory members of the intestinal microbiota. *Helicobacter hepaticus* is a normal member of the microbiota in many mouse facilities. *Helicobacter hepaticus* and a related species *Helicobacter bilis* normally do not cause pathology, but can trigger inflammation in susceptible hosts.^{6, 130} In the T cell transfer model of colitis, disease severity was greatly increased in the presence of *Helicobacter hepaticus*.¹³¹ Further, mice with a T_{reg}-specific deletion of IL-10 do not normally present any spontaneous phenotype. When infected with *Helicobacter*, these mice develop spontaneous colitis.⁷⁵ Though *Helicobacter hepaticus* appears to drive inflammation, T_{reg} cells from animals infected with *Helicobacter hepaticus* appear to be more suppressive than T_{reg} cells from uninfected animals.¹³² Given the numerous effects of *Helicobacter spp.* on T_{reg} cells and IBD, it seems likely that the variable presence of *Helicobacter* in mouse colonies may be one potential explanation for inconsistencies in IBD studies between institutions.

In mice, segmented filamentous bacteria (*Candidatus arthromitus*) also play an important role in intestinal immune responses. SFB are able to penetrate the mucus layer and directly contact the epithelium, making them particularly equipped to impact host immune responses.¹²¹ SFB are approximately 25 times more prevalent in C57BL/6 mice from the vendor Taconic Farms than in C57BL/6 mice from Jackson Labs, providing sources for relatively SFB sufficient and deficient mice.¹³³ In the T cell transfer model of colitis, the addition of SFB to a specific pathogen free bacterial cocktail led to severe colitis.¹³⁴ Further studies determined that SFB likely promote inflammation by driving Th17 cell differentiation in the small intestine LP.^{133, 135, 136} Monocolonization of GF animals with

SFB also leads to IgA production and increased numbers of IEL in the gut.^{135, 137} SFB induction of Th17 cells appears to be dependent on SFB production of serum amyloid A and ATP, which act on LP DCs, that in turn drive Th17 differentiation.^{128, 138}

The impact of SFB on human IBD is unknown however, since this bacterium is rarely found in the human microbiota. This may be due to prevention of SFB colonization by specific human defensins. Indeed, transgenic expression of human defensin 5 in mice leads to a lack of SFB colonization and a subsequent elimination of Th17 cells in the intestinal LP.¹³⁶ One report suggests that in addition to the induction of Th17 cells, SFB may also play a role in differentiation of iT_{reg} cells.¹³⁵ However, others found SFB did not increase T_{reg} cells, suggesting the source of SFB may play a role.¹³³

Though T_{reg} cell numbers generally increase when overall bacterial load is decreased, several studies demonstrated that specific bacterial taxa may drive peripheral Foxp3 induction and/or T_{reg} cell proliferation. In fact, the colon T_{reg} TCR repertoire was shown to differ from the T_{reg} repertoire at other peripheral sites, and these TCR sequences were specific for members of the microbiota. Further, these TCRs were colitogenic when expressed in the absence of Foxp3. These results demonstrate a role for peripheral differentiation of iT_{reg} cells specific for the bacterial microbiota and provide exciting support for the hypothesized role of iT_{reg} cells in broadening tolerance to the “extended self” of commensal microorganisms.¹¹⁸

Specific bacterial species may play roles in iT_{reg} differentiation. Though GF mice lack T_{reg} cells in the colonic LP, the colonization of GF mice with a distinct combination of 46 strains of *Clostridium spp.* led to normal development of T_{reg} cells in the gut.¹³⁹ These *Clostridium*-colonized mice had greater levels of intestinal TGF-β1 and increased levels of CTLA4 and IL-10 expression in the intestinal T_{reg} pool. *Faecalibacterium prausnitzii* is one species included in the 46 *Clostridium spp.*¹³⁹ *F. prausnitzii* has been shown to induce IL-10 production and reduce severity of colitis in mouse models. Interestingly, this bacterium is decreased in the intestines of IBD patients and is associated with greater risk of disease recurrence in post-surgical Crohn’s disease patients.¹⁴⁰

Numerous lactic acid bacteria and *Bifidobacteria* are used as probiotics, and several strains have shown effects on T_{reg} cells. *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri* have all been shown to modulate inflammation in mouse models of disease.³⁹ *Lactobacillus reuteri* were specifically shown to increase the frequency of T_{reg} cells in the mLN and spleen of host animals.^{141, 142} *Bifidobacterium infantis* increase the frequency and number of T_{reg} cells in the spleen and PP and decrease intestinal inflammation induced by *Salmonella typhimurium* infection.¹⁴³ Consuming *B. infantis* also increased the percent of Foxp3⁺ T_{reg} cells in the peripheral blood of human subjects.¹⁴⁴ VSL#3 is a probiotic mixture of numerous *Lactobacillus* and *Bifidobacteria* designed for the management of IBD. Treatment of mice with VSL#3 increases the production of IL-10 and number of TGF-β1-producing T cells, though it is unknown whether these cells are Foxp3⁺. VSL#3 treatment also decreases the severity of a chemically-induced model of colitis.¹⁴⁵

Generally, the presence or absence of bacterial taxa only correlates with effects on the T_{reg} cell pool. Administered microbes may exert their effects by modifying other members of the microbiota, and not through direct interaction with the immune system. However, *Bacteroides fragilis* exhibits a distinct mechanism for direct modification of host immune development. Monocolonization of GF mice with *B. fragilis* leads to increased differentiation of IL-10-producing iT_{reg} cells.¹⁴⁶ This induction is completely dependent upon expression of a single *B. fragilis* molecule, polysaccharide A (PSA). Further, this PSA-dependent iT_{reg} cell development requires TLR2 signaling, suggesting *B. fragilis* modifies

the host APC to drive iT_{reg} cell differentiation. *B. fragilis*-produced PSA inhibits Th17 induction and promotes iT_{reg} development, thereby protecting the host from both chemically-induced and *H. hepaticus*-driven colitis.^{146, 147} Conversely, enterotoxigenic strains of *B. fragilis*, which naturally lack PSA expression, play a role in inducing Th17 cells and promote intestinal inflammation.¹⁴⁸

Numerous bacterial strains can affect the host T_{reg} cell compartment. Many of these bacteria are promising candidates for probiotic or antibiotic treatments for IBD. However, most of these taxa, including *Lactobacillus*, *Bifidobacterium*, *Helicobacter*, *F. prausnitzii*, and *B. fragilis* are of low or variable abundance in the human microbiota, and SFB are largely absent in humans.^{39, 121} Further, probiotic and antibiotic-driven changes in the microbiota often do not endure once treatment is discontinued. Therefore, additional studies will be necessary to identify the role indigenous bacteria play on the T_{reg} cell lineage, and how these interactions may promote or prevent IBD.

Failure of regulatory mechanisms in IBD

Since IBD involves a failure in immune regulation, it seems likely that IBD is caused in part by defects in T_{reg} cells. These defects could be T_{reg} cell-intrinsic, resulting from inherently defective T_{reg} cells in the patient. Alternatively, the causes of defective T_{reg} cells could be T_{reg} cell-extrinsic, and a function of the abnormal intestinal environment and cytokine milieu in IBD patients. These T_{reg}-intrinsic or extrinsic defects can lead to insufficient T_{reg} cell suppression of IBD in at least 4 ways: 1) deficient T_{reg} cell numbers, 2) defective T_{reg} cell function, 3) unstable T_{reg} cell phenotype, and 4) pathogenic T cell resistance.¹⁴⁹

The majority of what we know about the mechanisms of T_{reg} involvement in IBD comes from mouse models. Mouse models allow better identification and tracking of *bona fide* T_{reg} cells and easier analysis of the intestinal milieu and sites of inflammation. Complete T_{reg} cell deficiency, such as that in scurfy or other Foxp3 mutant mice, leads to fatal autoimmunity that includes intestinal inflammation, underscoring deficient T_{reg} number as a potential mechanism for IBD.^{8, 150} In T cell transfer models of colitis the colitogenic T cell population must be depleted of T_{reg} cells in order to cause disease.¹⁹ Further, injection of both T_{reg} cell subsets into these mice can prevent or cure IBD in a dose-dependent fashion.³⁸ Mutations in other potentially T_{reg} cell-intrinsic genes such as CD25, IL-2, CD28, and TGF-β1 also lead to decreased numbers of T_{reg} cells, though IL-2 and TGF-β1 expression by other cell types likely contributes.^{2, 149} Other T_{reg} cell-extrinsic factors also play a role in mouse models. Cytokines such as IL-6, IL-21, IL-27, and IL-23 may suppress differentiation of iT_{reg}, suggesting a role for the intestinal milieu on T_{reg} cell number.⁶

T_{reg} cell function may also be affected by the same cytokines that lead to changes in T_{reg} cell number. In addition, other T_{reg} cell-extrinsic cytokines such as TNF, IL-4, IL-12, IL-7, and IL-15 may limit T_{reg} function.¹⁴⁹ Several molecules have been shown to play T_{reg} cell-intrinsic roles in T_{reg} suppressive function. Mutations in T_{reg} surface receptors CTLA-4, CD39, LAG-3, and Fas have been shown to limit T_{reg} cell suppression.¹⁴⁹ In addition, T_{reg} cells deficient in the soluble factors TGF-β1, IL-10, and IL-35 have decreased function (Table 2).¹⁴⁹

Mouse T_{reg} cells have also shown variable stability. IL-6 exposure drives a fraction of T_{reg} cells into the Th17 lineage *in vitro*.¹⁵¹ In the T cell transfer model of colitis, transfer of T_{reg} cells into an IL-10 deficient host led to a loss of Foxp3 expression and conversion of T_{reg} cells into Th1 pathogenic effector cells.¹⁵² Adoptive transfer of T_{reg} cells into T cell deficient hosts led to the loss of Foxp3 expression and conversion of T_{reg} cells into T follicular helper cells in the intestinal PP.¹⁵³ Other lineage-tracing studies showed that T_{reg} cells that lost Foxp3 expression converted to Th17 cells in the PP, but became more Th1-

like in the spleen and mLN. Further, these studies showed that in a mouse model of diabetes T_{reg} cells that lost Foxp3 expression could be pathogenic upon re-transfer.¹⁵⁴ In the transfer model of colitis, the transfer of *in vitro*-derived iT_{reg} cells as treatment resulted in 85% of transferred cells losing Foxp3 expression, becoming $ex-iT_{reg}$ cells with pathogenic potential.¹¹⁷ Conversely, another study utilizing an inducible cell-tracking system found T_{reg} to be rather stable.¹⁵⁵ The differences in these studies may reflect the differences in iT_{reg} cell vs nT_{reg} cell involvement in each system. In general, these studies have shown the iT_{reg} cell lineage to be more plastic and able to become pathogenic $ex-iT_{reg}$, whereas the nT_{reg} cell lineage demonstrates greater stability (Figure 1).

Mouse models have shown that even in the presence of functional T_{reg} cells, pathogenic effector cells can be resistant to T_{reg} -mediated suppression. In addition to affecting T_{reg} cell function, the cytokines IL-2, IL-4, IL-7, and IL-15 can drive proliferation of effector T cells in the presence of T_{reg} cells *in vitro*.¹⁵⁶ Also, agonistic stimulation of TNF receptor family members OX40 or 4-1BB on conventional T cells leads to effector T cells that are resistant to T_{reg} suppression *in vitro*.^{157, 158} For OX40, this action has been demonstrated *in vivo* in the T cell transfer model of colitis.¹⁵⁸

Human studies of IBD have a distinct advantage over other autoimmune diseases such as multiple sclerosis and lupus because tissue samples from the site of inflammation can be removed and studied. However, since human activated effector T cells also express Foxp3, histological analysis of T_{reg} cells in IBD patients can be difficult. Nonetheless, studies with human patients have been able to support regulatory mechanisms first discovered in mouse models. Research in human patients has similarly investigated T_{reg} cell number, T_{reg} cell function, T_{reg} cell stability, and effector cell resistance to T_{reg} as potential causes of IBD.

Numerous studies have investigated T_{reg} cell number in human IBD patients. In general, these reports observe greater numbers of Foxp3⁺ cells in the intestine of patients, particularly in active inflammatory lesions.^{77, 159–164} Numbers of T_{reg} cells in the peripheral blood are less consistent than the intestine. However, most studies identify a decreased frequency of T_{reg} cells in the peripheral blood of IBD patients. This decrease is intensified as T_{reg} cell numbers increase in the intestine during active disease, suggesting a potential redistribution of these cells in response to active inflammation.^{77, 162–165} Interestingly, the increase in T_{reg} cells in the intestine does not appear to be specific to autoimmune disease, as similar or greater numbers of T_{reg} cells are seen in other inflammatory conditions such as diverticulitis and enteritis.^{77, 162}

T_{reg} cells at the site of inflammation fail to protect against IBD despite being present in increased numbers. This suggests that T_{reg} function may be compromised in these patients. To investigate this point, several groups removed T_{reg} cells from IBD patients and tested their suppressive function *in vitro*. These studies showed uniformly that T_{reg} cells from the peripheral blood, mLN, and LP of IBD patients were all equally suppressive to T_{reg} cells from normal controls.^{159, 161–163, 166–168} Thus, T_{reg} cells from IBD patients appear to be present in sufficient numbers and function normally *ex vivo*. These results suggest that there may not be an intrinsic defect of T_{reg} cells in IBD, but an extrinsic effect of the intestinal milieu on T_{reg} cells. In fact, numerous IBD susceptibility genes such as autophagy genes, NOD2, and IL23R, could play a role in creating an abnormal intestinal microenvironment that limits T_{reg} cell function *in vivo*. Previous studies in mouse models suggest that cytokines within an inflammatory microenvironment may also promote T_{reg} cell instability.¹⁶⁹ Similar results have been seen for human T_{reg} cells. Human T_{reg} cells from peripheral blood can convert to Th17 cells when stimulated *in vitro* in the presence of IL-2. This conversion is increased by inflammatory cytokines IL-1 β , IL-23, and IL-21 or APCs activated by microbial stimuli.¹⁷⁰

Effector T cell hyporesponsiveness to suppression is another T_{reg} cell-extrinsic factor that may play a role in human IBD. Two studies investigated T_{eff} responsiveness and found no difference between IBD patients and controls.^{159, 167} Other groups found T_{eff} cells from patients to be resistant to T_{reg} suppression. These studies suggested that T_{eff} cells from IBD patients overexpress SMAD7, thus making them resistant to TGF- β 1 secreted by T_{reg} cells.^{42, 171} Further, T_{reg} cells may suppress disease by driving T_{eff} apoptosis, and T_{eff} cells in IBD patients are generally resistant to apoptosis.^{172, 173} Given the data suggesting a potential role for the intestinal milieu, it is important to note that these studies of T_{eff} responsiveness are performed on T cells outside of the inflammatory intestinal niche.

The potential for T_{reg} cells in IBD therapies

Since T_{reg} cells play a requisite role in preventing autoimmunity, these cells may be ideal targets for developing new treatments for IBD. There are currently over 50 products being tested for use in the treatment of IBD. These include small molecules, siRNA, peptides, vaccines, and numerous monoclonal antibodies and cell-based treatments.⁹⁸ Several of these treatments center on T_{reg} cells. However, additional research has suggested several other potential mechanisms for T_{reg}-based therapies. In order to find new therapies that rely on T_{reg} cells researchers must characterize T_{reg} cells during the disease process, and determine the mechanisms of impaired T_{reg} cell suppression in IBD. This knowledge will aid in developing treatments that will increase T_{reg} cell number, function, and stability.¹⁴⁹ Developing treatments involving T_{reg} cells is particularly challenging for IBD since the precise role for T_{reg} cells in IBD patients has not been fully elucidated.

Traditional treatments for IBD appear to have effects on the T_{reg} cell compartment. Standard treatment of UC patients with aminosalicylates or glucocorticoids led to increased peripheral blood frequencies of CD4⁺CD45RO⁺CD25⁺ T cells, a population enriched in T_{reg} cells.¹⁶⁴ In CD however, treatment with azathioprine or mercaptopurine led to decreased frequencies of T_{reg} cells in the peripheral blood.¹⁶³ Treatment with monoclonal antibodies to TNF- α (infliximab) was shown to increase T_{reg} cells in the peripheral blood and intestinal LP by two separate groups.^{174, 175} However, another group did not see any difference in T_{reg} cell frequency upon infliximab treatment.¹⁷⁶ These classically used IBD treatments have broad effects, and a disadvantage of these approaches is down-modulation of both innate and adaptive immunity and increased risk for opportunistic infection.⁴⁸ Therefore, there is a need to increase the specificity of IBD treatments, with T_{reg} cells as one particular area of interest.

IBD treatments focused on T_{reg} cells fall into two categories: cell-based treatments that involve the transfer of *in vitro* expanded or stimulated T_{reg} cells into patients, or pharmacological approaches that attempt to influence T_{reg} cells *in vivo*. The small number of T_{reg} cells that can be obtained from a patient's blood limits cell-based therapies. Thus numerous approaches are being investigated to expand and activate a patient's own peripheral blood T_{reg} cells for re-transfer. Such approaches have been shown to increase the number of T_{reg} cells up to 13000 fold in 3–4 weeks. Yet, a fraction of these cells lose Foxp3 expression, indicating instability of the T_{reg} cells or outgrowth of T_{conv} cells in this population.⁶¹ Another potential source for T_{reg} cell-based treatments is the *in vitro* differentiation of iT_{reg} cells from T_{conv} precursors via TCR stimulation and TGF- β 1. This approach can yield an average of 240×10^9 iT_{reg} cells in approximately 2 weeks in culture.¹⁷⁷

Both *in vitro* expansion of nT_{reg} and differentiation of iT_{reg} are limited by several factors. These T_{reg} cells may still have relatively few antigen specificities or the incorrect antigen specificities for proper suppression. Tetramer-based approaches could lead to expansion of

relevant T_{reg} cell specificities, but rely on knowledge of the target antigens for disease.¹⁷⁸ Further, transferred T_{reg} cells may harbor potentially harmful specificities if they do not remain stably suppressive.^{117, 154} Addition of rapamycin to cultures leads to increased numbers of T_{reg} cells for both *in vitro* nT_{reg} expansion and iT_{reg} differentiation.^{177, 179} Further, rapamycin reduces the outgrowth of contaminating effector T cells, and increases T_{reg} stability.¹⁸⁰ Another limitation of cell-based approaches is that transferred T_{reg} cells may not localize to the inflamed target tissue. T_{reg} cells could be engineered with receptors such as tissue-specific integrins, driving a T_{reg} cell to home the intestine. However, for these approaches to work in human patients gene therapy would have to be utilized, which raises safety concerns.

Mouse studies have utilized T_{reg} cells that become activated by a bystander Ag in the target tissue to increase T_{reg} localization.¹⁸¹

One recent phase 1/2a clinical study demonstrated both safety and efficacy of ovalbumin-specific T_{reg} cells in a small CD patient population.¹⁸² Autologous T_{reg} cells were expanded *in vitro*, cloned by limiting dilution, and selected for IL-10 production in response to ovalbumin. Since ovalbumin is a common food antigen, ovalbumin specificity was used as a bystander antigen in an attempt to drive gut homing and local immune regulation in the intestine. Five weeks post-treatment 8/20 patients showed significant clinical improvement, with 6/8 showing improvement at one dose. However, the T_{reg} subset being used in these studies is indeterminate since they express numerous molecules associated with Tr1 cells, yet are activated *in vitro* and can also express Foxp3.¹⁸²

Cell-based treatments may also utilize populations other than T_{reg} cells. Transfer of tolerogenic DCs could drive iT_{reg} differentiation *in vivo*.¹⁸³ Mesenchymal stem cells can produce IL-10 and TGF-β1, which could lead to increased iT_{reg} differentiation or overall T_{reg} function.^{98, 184} Transfer of mesenchymal stem cells ameliorated a mouse model of chemically induced colitis and more than doubled the frequency of T_{reg} cells in the mLN of treated mice.¹⁸⁵

Recent studies underscore the need for both nT_{reg} and iT_{reg} cells for complete tolerance.²⁴ Thus, clinical studies may need to utilize transfer of both nT_{reg} and iT_{reg} cells. Alternatively, nT_{reg} cells could be transferred in addition to T_{conv} cells that cannot become pathogenic, or nT_{reg} cells in combination with another cell population to drive *in vivo* iT_{reg} differentiation. One can also imagine utilizing either nT_{reg} or iT_{reg} cells for different clinical outcomes.³⁵ For example, an inflammatory response against the extended self may benefit more from iT_{reg} cell specificities, whereas an autoimmune response may be better treated via nT_{reg} cells. However, as noted T_{reg} cell numbers are not generally decreased in human patients, and T_{reg} cells from these patients are generally suppressive *ex vivo*. Thus, T_{reg} cells in IBD patients may not be intrinsically flawed, but may fail to suppress disease due to local factors affecting T_{reg} cell function. Thus, other therapeutic approaches may be necessary to increase T_{reg} cell number or function *in situ*.

Probiotics are one approach to increase T_{reg} cell responses specifically in the intestine. Since IBD patients often display an abnormal microbiome, reshaping of the intestinal microbiota has been suggested as a potential treatment for disease. In fact, complete microbiota transplant, also known as fecal bacteriotherapy, has been used to treat *Clostridium difficile*-associated colitis, and has shown promise in some cases of IBD.^{123, 186, 187} More commonly, specific species or of bacteria are utilized as probiotics. Many of these treatments were found to increase T_{reg} cell numbers and function in both humans and mice, likely through local effects on gut-associated DCs.^{144, 188–190} Probiotic strains can also be genetically engineered to better modify the inflammatory intestinal milieu.¹⁹¹ *Lactococcus*

lactis engineered to secrete IL-10 transiently colonized mice and decreased two models of colitis.⁷² A phase I clinical trial demonstrated safety of a similar approach in human patients, but efficacy has not been determined.¹⁹²

Vitamins A and D may also affect T_{reg} cells and serve as potential therapeutic agents for IBD. The vitamin A metabolite RA appears to increase T_{reg} cell induction and homing in the intestine, and RA treatment ameliorated mouse models of IBD.¹⁹³ Further, RA treatment increases Foxp3 expression in human intestinal biopsies, suggesting potential therapeutic applications for vitamin A metabolites in IBD.¹⁹³ Vitamin D is a sunlight-dependent precursor to the hormone calcitriol. Vitamin D metabolites and analogues have been variably shown to stimulate mouse and human T_{reg} cell and T_{eff} differentiation and function.^{194–196} Interestingly, IBD risk increases significantly with increasing latitude, suggesting a role for vitamin D as an environmental risk factor in IBD.¹⁹⁷ Also, higher predicted serum levels of vitamin D reduce the risk for IBD, and the vitamin D receptor is a potential susceptibility allele for UC and CD.^{198, 199} Numerous mouse studies also support a protective role for vitamin D in IBD.²⁰⁰ However, few intervention studies treating IBD patients with vitamin D have been performed. A recent meta-analysis suggested a potential benefit to vitamin D supplementation of IBD patients, but underscored the need for randomized placebo-control studies.²⁰¹

In atopic disease allergen-specific hyposensitization is a common approach for inducing tolerance to an antigen. This involves repeated parenteral or mucosal vaccination with the antigen at increasing doses, and has been shown to be safe and decrease hypersensitivity. Significantly, this approach seems to rely on the differentiation or activation of antigen-specific T_{reg} cells.⁷ These approaches have been used for food allergy, suggesting this approach is effective for intestinal antigens.²⁰² Hyposensitization may be difficult to apply to IBD since the specific antigens for this hypersensitivity are largely unknown. Nevertheless, a recent study in a mouse model showed promising results. Oral administration of components of one specific species of the microbiota led to increased T_{reg} cell number and frequency in the mLN. Further, this treatment prevented a chemically induced model of colitis, and attenuated established disease.²⁰³

As mentioned, the classical treatments of IBD may obtain some of their beneficial results via effects on T_{reg} cells. However numerous other pharmacologic agents are also used to treat IBD, and often have notable effects on T_{reg} cells. In mouse models, histone deacetylase inhibitors increased T_{reg} cell number and function, and decreased chemically induced colitis.^{204, 205} Other small molecules such as siRNA and nanoparticles are currently being used to target intestinal tissue, and may also have future applications to increase T_{reg} cell number and/or function in the gut.⁹⁸

Monoclonal antibodies are the largest class of pharmaceuticals in development for treatment of IBD. Anti-p40 (briakinumab, ustekinumab) limits Th17 and Th1 responses in IBD patients by blocking the cytokines IL-12 and IL-23.^{206–208} Anti-p40 may also have beneficial effects on T_{reg} cells since IL23Rko mice have increased T_{reg} cell numbers, particularly in the GI tract.^{209, 210} Anti-CD3 has shown beneficial effects in mouse models of autoimmune disease, leading to deletion and anergy of pathogenic T cells, while promoting T_{reg} cell responses.²¹¹ However, one anti-CD3 monoclonal antibody (visilizumab) recently failed to show efficacy after parenteral injection, despite promising pilot studies.²¹² Clinical studies treating IBD patients with an oral anti-CD3 antibody (muromonab) are currently underway.²¹³

Other integrin-specific monoclonal antibodies are being used to limit pathogenic T cell access to intestine. Natalizumab, anti- α 4 integrin (CD49d) has been approved for use in IBD

in the U.S. However, unexpected complications arose with this drug due to $\alpha 4$ integrin's role in lymphocyte homing to the CNS.²¹⁴ Thus, newer studies are utilizing vedolizumab to target the $\alpha 4\beta 7$ integrin dimer, which appears to control homing only to the intestine.^{215, 216} One concern with monoclonal antibodies targeting integrins is that they may also limit T_{reg} cell access to the site of inflammation. However, any constraints on T_{reg} access to the intestine may be counteracted by the blockade of pathogenic T cells and the ability of T_{reg} cells to suppress autoimmune responses in the mLN and other peripheral lymphoid tissues.

In general, the development of potential treatments for IBD is outpacing the ability to test these approaches in human patients. Clinical studies are often statistically limited by the ability of a small number of willing patients to differentiate relatively small effects on disease progression.⁹⁸ Future studies may benefit greatly from personalized medicine approaches to stratify treatment groups in a clinical study. Genetic screens and serum biomarker analysis may identify the patients most likely to respond to a T_{reg}-based therapy. Examination of a patient's microbiota, and other non-genetic factors such as nutritional and vitamin status may also increase the success of treatments. Exciting new treatments from IBD will likely result from combining the increased ability to develop novel therapies with new technologies to identify the patients most likely to respond. A combination of genetics, microbiota, and other non-genetic/environmental influences are thought to contribute to IBD risk. Importantly, T_{reg} cells interact with, and can be affected by all three of these contributing factors. It therefore seems highly likely that future treatments for IBD will include therapies targeting and capitalizing on the potent immunomodulatory effects of T_{reg} cells.

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Figure 1.

Model of T_{reg} cell development. The nT_{reg} (green) and T_{conv} (grey) cell populations develop as separate lineages in the thymus, based in part on differences in the threshold for affinity-based selection. The result is a peripheral population of nT_{reg} and T_{conv} cells with distinct TCR (orange) repertoires. In the gut and at other environmental interfaces, TGF- β 1 induces T_{conv} cells to become iT_{reg} cells (red) or $Th17$ cells (purple). The peripheral T_{reg} cell pool is therefore comprised of both iT_{reg} and nT_{reg} cells that share suppressive mechanisms such as IL-10 production. Increased levels of IL-6 in the local environment promote production of $Th17$ cells while blocking iT_{reg} cell formation. In general, iT_{reg} cells are unstable and may lose Foxp3 expression ($ex-iT_{reg}$ cells), although certain factors may increase iT_{reg} stability.

The ex- iT_{reg} cells (pink) are available to become Th1 (blue) or Th17 cells, or to reacquire Foxp3 expression and cycle back into the iT_{reg} pool. Importantly, nT_{reg} and iT_{reg} cells act synergistically and are non-redundant, a feature based largely on their different TCR repertoires.

Table 1T_{reg} cell involvement in commonly used mouse models of IBD

Category of IBD Model	Model Name	Role for Regulatory T cells	References
1) Barrier disruption	DSS (chemical-induced)	- Absence of T _{reg} or TGF-β1 signaling in T _{reg} cells increases severity	20,217
	TNBS/DNBS/Oxazolone (hapten-induced)	- Ethanol in absence of TNBS leads to resistance	218
		- <i>B. fragilis</i> PSA increases iT _{reg} cells leading to resistance	146
		- T _{reg} cells transiently decrease then home to intestine during remission	219
2) Genetic deficiency	IL-10 deficiency	- T _{reg} -produced IL-10 is necessary to prevent intestinal inflammation	75, 76, 77
	SAMP/YitFc (<i>PPARG</i> mutation)	- T _{reg} cells from these mice are defective in suppressing colitis upon transfer	16, 220
	Mdr1 deficiency	- mutation leads to decreased <i>in vivo</i> and <i>in vitro</i> differentiation of iT _{reg}	17
3) Overexpression of inflammatory mediators	TNF ^{ΔARE} (TNF-α overexpression)	- increased differentiation of iT _{reg} attenuates disease	221
	STAT4 overexpression	- unknown, T _{reg} cells hypothesized to be normal	21
4) Lymphopenic T cell transfer	Naïve T _{conv} transfer into SCID/ RAG ^{-/-} mice	- co-transfer of nT _{reg} cells prevents disease	19
		- nT _{reg} + <i>in vivo</i> or <i>in vitro</i> iT _{reg} cells treat disease	38

Table 2T_{reg} cell suppressive mechanisms in IBD

	Regulatory Mechanism	Utilized by		Role in IBD		References
		nT _{reg}	iT _{reg}	Mouse Models	Human Patients	
Soluble Mediators	TGF- β 1	Yes	Yes	Yes	Yes	42, 222
	IL-10	Yes	Yes	Yes	Yes	12, 73–75, 117
	IL-35	Yes	No	Yes	Unknown	79, 223
	Pericellular adenosine (CD39/CD73)	Yes	Unknown	Yes	Unknown	82–85
Cell-Cell Interaction	Sink for IL-2 (CD25 ^{hi})	Yes	Yes	Yes	Yes	22, 33, 36, 83, 87
	Cytotoxicity (perforin/granzymes)	Yes	Yes	Unknown	Unknown	89, 90
	CTLA-4	Yes	Yes	Yes	Yes	95, 98, 224, 225
	LAG-3	Yes	Unknown	Yes	Unknown	99, 226, 227
	PD-1/PD-L1	Yes	Yes	Yes	Unknown	100
	Nrp-1	Yes	No	Unknown	Unknown	54, 55
	Galectins	Yes	Unknown	Yes	Yes	228–230
	GITR	Yes	Yes	Yes	Unknown	231, 241
	OX40	Yes	Yes	Yes	Unknown	232, 233